SUPPLEMENTARY INFORMATION

Design and Characterization of Bivalent BET Inhibitors

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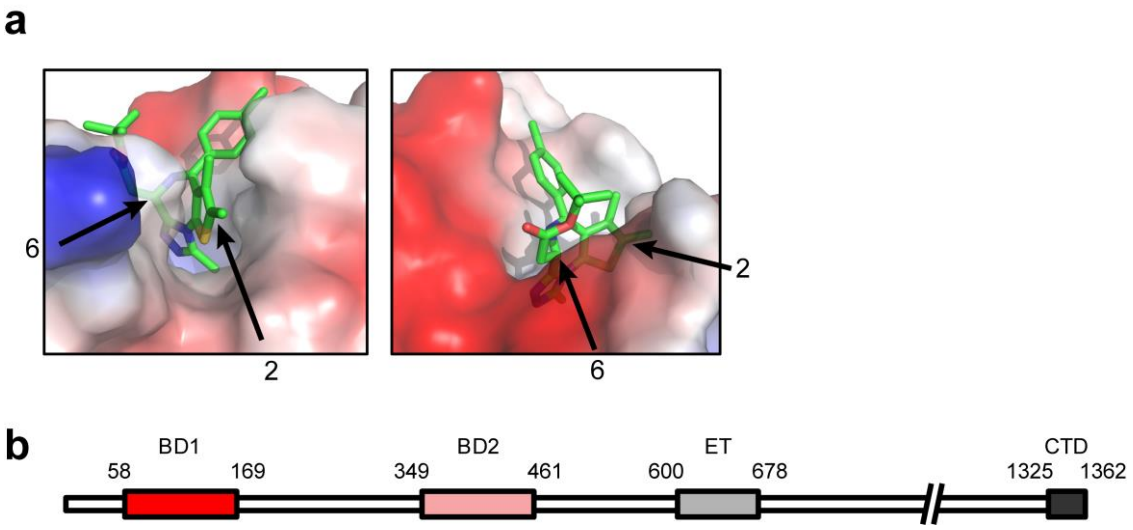
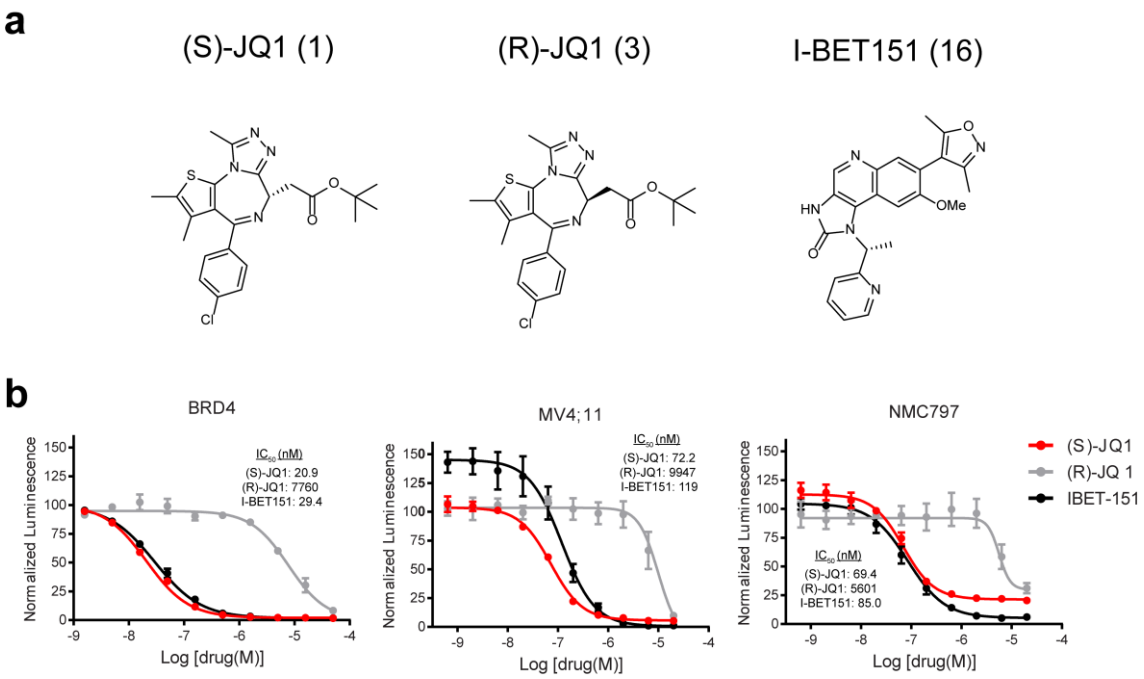
SUPPLEMENTARY RESULTS

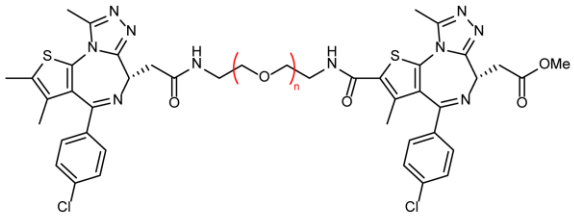


Supplementary Figure 1 | Structural basis of bivalent BET inhibitor design. (**a**) Crystal structure of BRD4(1) with JQ1 (PDB: 3MXF). Positions amenable to chemical modification are indicated by arrows. (**b**) Primary structure of human BRD4 with domains shown.



Supplementary Figure 2 | Activities of (*S*)- and (*R*)-JQ1 and I-BET151. (**a**) Chemical representation of the *S* and *R* enantiomers of JQ1 and I-BET151. (**b**) Biochemical and cellular activities of the *S* and *R* enantiomers of JQ1 and I-BET151. Biochemical and cellular data points represent the mean of at least 2 or 4 replicates respectively ± SD.

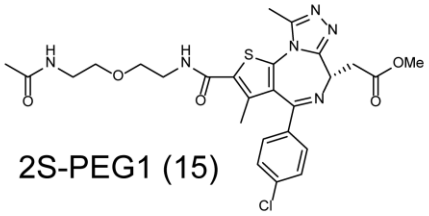
a



b

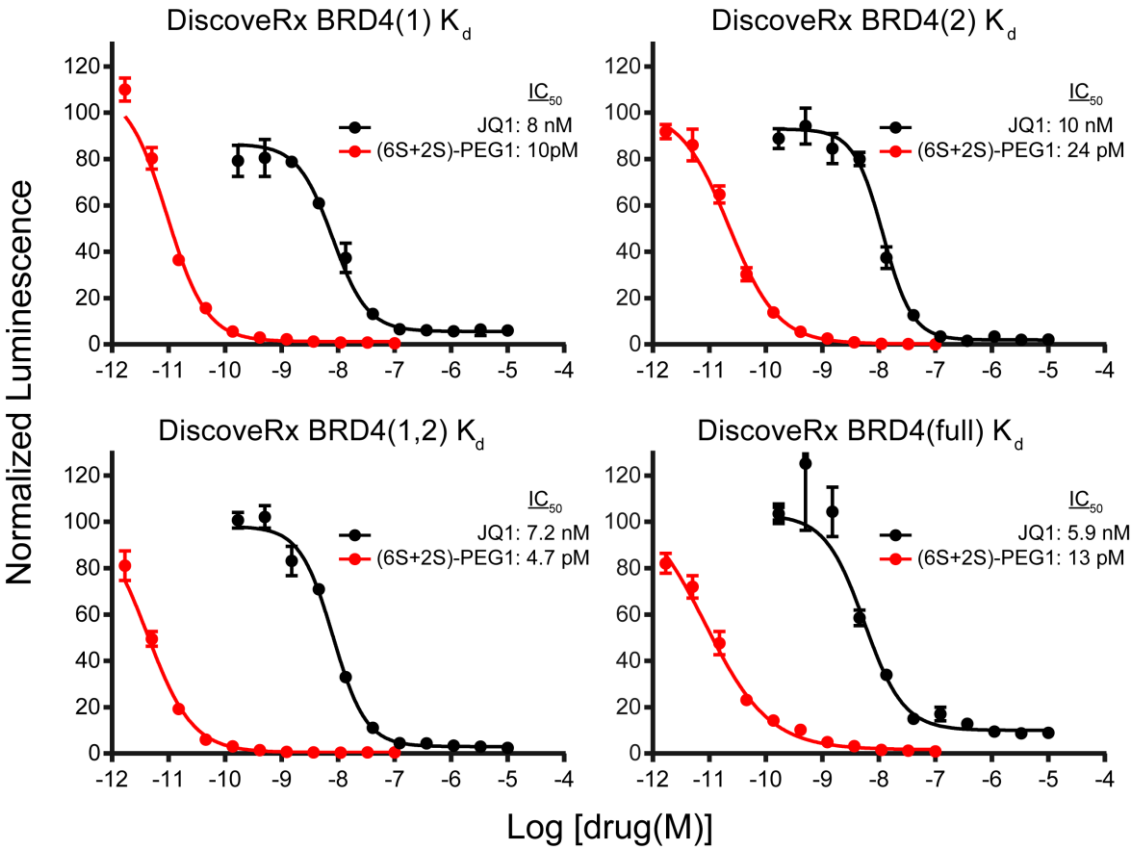
		Properties			Alpha; IC_{50} (nM)		BROMOscan; K_d (nM)					Cell; IC_{50} (nM)	
ID	n	MW	cLogP	tPSA	BRD4(1)	BRDT(1)	BRD4(1)	BRD4(2)	BRD4(1,2)	BRD4(full)	BRDT(1,2)	NMC797	MV411
(6S+2S)-PEG0 (6)	0	870	4.9	165	3.58	2.36	0.034	NT	0.047	NT	NT	0.736	0.358
(6S+2S)-PEG1 (7)	1	914	5.0	174	0.758	0.574	0.01	0.024	0.0047	0.013	0.039	1.15	0.236
(6S+2S)-PEG2 (8)	2	958	4.8	184	1.28	1.84	0.014	NT	0.0091	NT	NT	1.06	0.216
(6S+2S)-PEG3 (9)	3	1002	4.6	193	2.25	0.878	0.029	NT	0.0075	NT	NT	0.704	0.308
(6S+2S)-PEG4 (10)	4	1046	4.4	202	2.95	1.09	0.023	NT	0.0066	NT	NT	1.39	0.998
(6S+2S)-PEG7 (11)	7	1178	3.9	230	0.789	1.59	0.026	NT	0.0059	NT	NT	0.614	2.559

c



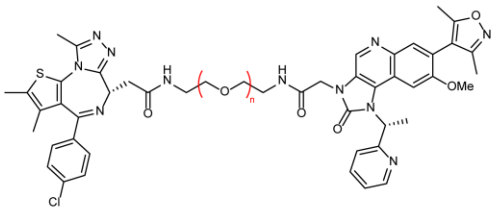
2S-PEG1 (15)

Supplementary Figure 3 | SAR of (6+2)-PEG analogs. (**a**) Chemical representation of (6+2)-PEG analogs with different linker lengths (n). (**b**) SAR properties of (6+2)-PEG analogs including molecular weight (MW), logarithm of the partition coefficient (cLogP), the total polar surface area (tPSA) and biochemical and cellular potencies. (**c**) Chemical structure of single warhead with PEG1 linker (2S-PEG1). NT: not tested.



Supplementary Figure 4 | BROMO*scan*™ of (6S+2S)-PEG1 and JQ1. (**a**) Potency of hetero-dimeric compounds was evaluated by BROMO*scan*™ (DiscoverX). Data points represent the mean of 2 replicates ± SD.

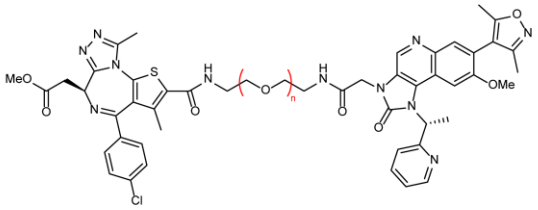
a



b

		Properties			Alpha; IC_{50} (nM)		BROMOscan; K_d (nM)					Cell; IC_{50} (nM)	
ID	n	MW	cLogP	tPSA	BRD4(1)	BRDT(1)	BRD4(1)	BRD4(2)	BRD4(1,2)	BRD4(full)	BRDT(1,2)	NMC797	MV411
6S+IBET-PEG0 (17)	0	898	5.0	178	404	148	NT	NT	NT	NT	NT	465	201
6S+IBET-PEG1 (18)	1	942	5.1	187	54	12.1	NT	NT	NT	NT	NT	22	12
6S+IBET-PEG2 (19)	2	987	4.9	196	58.6	10	0.062	NT	NT	NT	NT	61	14
6S+IBET-PEG3 (20)	3	1031	4.7	205	27.4	5.78	NT	NT	NT	NT	NT	96	16
6S+IBET-PEG4 (21)	4	1075	4.6	215	29.5	6.2	NT	NT	NT	NT	NT	196	10
6S+IBET-PEG7 (22)	7	1207	4.0	242	11.5	6.1	NT	NT	NT	NT	NT	150	8.5

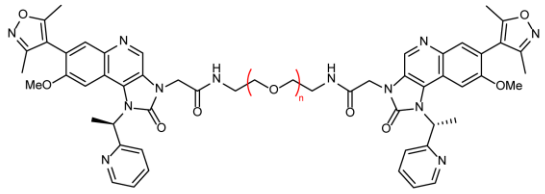
c



d

		Properties			Alpha; IC_{50} (nM)		BROMOscan; K_d (nM)					Cell; IC_{50} (nM)	
ID	n	MW	cLogP	tPSA	BRD4(1)	BRDT(1)	BRD4(1)	BRD4(2)	BRD4(1,2)	BRD4(full)	BRDT(1,2)	NMC797	MV411
2S+IBET-PEG0 (23)	0	942	4.0	204	439	596	NT	NT	NT	NT	NT	4,206	3,053
2S+IBET-PEG1 (24)	1	987	4.1	213	18.1	40.3	NT	NT	NT	NT	NT	555	167
2S+IBET-PEG2 (25)	2	1031	3.9	222	5.8	23.2	NT	NT	NT	NT	NT	291	13
2S+IBET-PEG3 (26)	3	1075	3.7	232	11.3	31.7	NT	NT	NT	NT	NT	352	17
2S+IBET-PEG4 (27)	4	1119	3.5	241	10.3	21.7	NT	NT	NT	NT	NT	302	19
2S+IBET-PEG7 (28)	7	1251	3.0	269	17.4	13.2	NT	NT	NT	NT	NT	457	33

e



f

		Properties			Alpha; IC_{50} (nM)		BROMOscan; K_d (nM)					Cell; IC_{50} (nM)	
ID	n	MW	cLogP	tPSA	BRD4(1)	BRDT(1)	BRD4(1)	BRD4(2)	BRD4(1,2)	BRD4(full)	BRDT(1,2)	NMC797	MV411
IBETx2-PEG0 (29)	0	971	3.2	217	209	203	NT	NT	NT	NT	NT	2,549	345
IBETx2-PEG1 (30)	1	1015	3.3	226	108	203	NT	NT	NT	NT	NT	3,393	315
IBETx2-PEG2 (31)	2	1059	3.1	235	117	123	NT	NT	NT	NT	NT	6,146	330
IBETx2-PEG3 (32)	3	1103	2.9	244	53.4	64.6	NT	NT	NT	NT	NT	3,313	348
IBETx2-PEG4 (33)	4	1147	2.7	253	73.7	60.2	NT	NT	NT	NT	NT	3,026	249
IBETx2-PEG7 (34)	7	1279	2.2	281	30.5	13.6	NT	NT	NT	NT	NT	542	92

Supplementary Figure 5 | SAR of hetero- and homo-dimers of I-BET151 and JQ1. (**a**, **c**, **e**) Chemical representation of hetero- and homo-dimers of I-BET151 and JQ1 with different linker lengths (n). (**b**, **d**, **f**) SAR properties of hetero- and homo-dimers of I-BET151 and JQ1 including molecular weight (MW), logarithm of the partition coefficient (cLogP), the total polar surface area (tPSA) and biochemical and cellular potencies. NT: not tested.

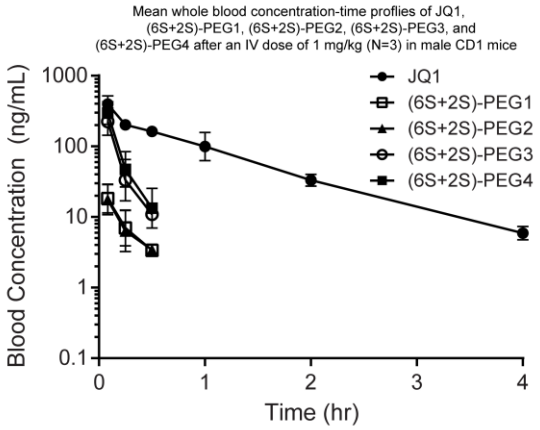
a

(6S+2S)-PEG1
BRD4(1): 0.758 nM
NMC797: 1.15 nM
MV4;11: 0.236 nM

(6S+2S)-PEG2
BRD4(1): 1.28 nM
NMC797: 1.06 nM
MV4;11: 0.216 nM

(6S+2S)-PEG3
BRD4(1): 2.25 nM
NMC797: 0.704 nM
MV4;11: 0.308 nM

(6S+2S)-PEG4
BRD4(1): 2.95 nM
NMC797: 1.39 nM
MV4;11: 0.998 nM



Pharmacokinetic parameters of JQ1, (6S+2S)-PEG1, (6S+2S)-PEG2, (6S+2S)-PEG3 and (6S+2S)-PEG4 after IV administration at 1 mg/kg in male CD1 mice

Dose (mg/kg)	Dose route	PK parameters (unit)	JQ1	(6S+2S)-PEG1	(6S+2S)-PEG2	(6S+2S)-PEG3	(6S+2S)-PEG4
1	IV	CL (L/hr/kg)	3.34	112	115	10.5	10.9
	N=3	V_{ss} (L/kg)	3.01	20.7	22.1	0.974	0.877
		AUC_{last}(hr*ng/mL)	307	4.98	4.90	59.7	78.7
		AUC_{INF}(hr*ng/mL)	313	8.93	8.68	95.4	97.0
		Terminal $t_{1/2}$ (hr)	0.788	0.137	0.141	0.0835	0.0857
		MRT_{INF}(hr)	0.897	0.185	0.192	0.0930	0.0850

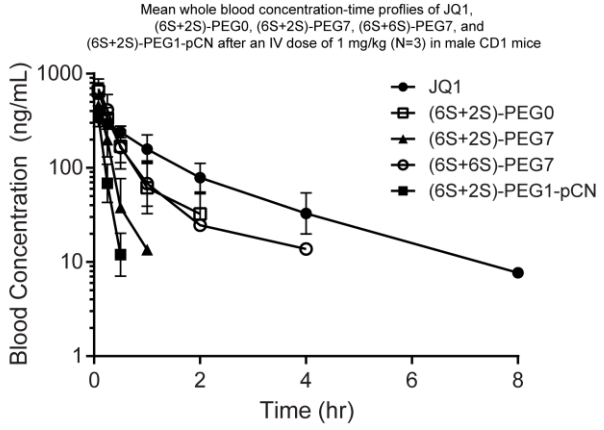
b

(6S+2S)-PEG0
BRD4(1): 3.58 nM
NMC797: 0.736 nM
MV4;11: 0.358 nM

(6S+2S)-PEG7
BRD4(1): 0.789 nM
NMC797: 0.614 nM
MV4;11: 2.56 nM

(6S+6S)-PEG7
BRD4(1): 3.09 nM
NMC797: 0.792 nM
MV4;11: 0.170 nM

(6S+2S)-PEG1-pCN
BRD4(1): 0.462 nM
NMC797: 5.76 nM
MV4;11: 1.38 nM

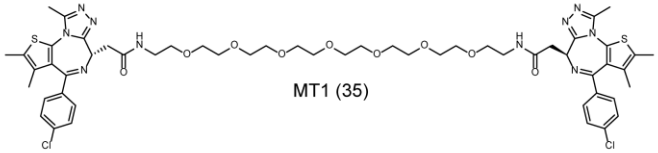


Pharmacokinetic parameters of JQ1, (6S+2S)-PEG0,(6S+2S)-PEG7,(6S+6S)-PEG7,(6S+2S)-PEG1-pCN after IV administration at 1 mg/kg in male CD1 mice

Dose (mg/kg)	Dose route	PK parameters (unit)	JQ1	(6S+2S)-PEG0	(6S+2S)-PEG7	(6S+6S)-PEG7	(6S+2S)-PEG1-pCN
1	IV	CL (L/hr/kg)	1.93	3.55	7.59	3.20	11.4
	N=3	V_{ss} (L/kg)	3.02	1.23	1.11	1.45	1.19
		AUC_{last}(hr*ng/mL)	520	296	142	359	95.0
		AUC_{INF}(hr*ng/mL)	557	312	145	369	95.6
		Terminal $t_{1/2}$ (hr)	1.33	0.332	0.114	0.594	0.0834
		MRT_{INF}(hr)	1.67	0.386	0.156	0.532	0.104

Supplementary Figure 6 | Pharmacokinetic analyses of bivalent inhibitors. (**a**, **b**) Plasma pharmacokinetic analyses of bivalent molecules performed as 5-in-1 cassette dosing (4 bivalent compounds and JQ1). Five compounds (1 mg/kg) were administered intravenously to CD1 mice (n = 3).

a

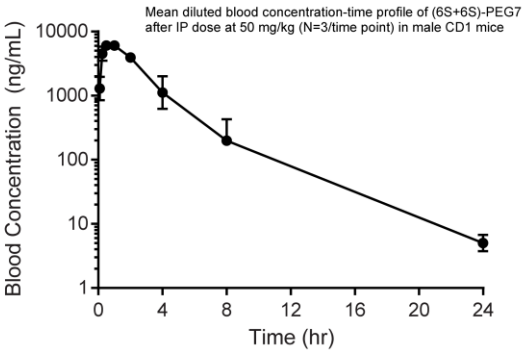


MT1 (35)

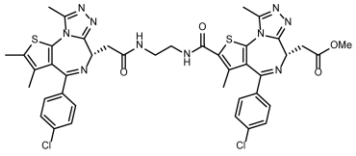
(6S+6S)-PEG7 (35) (50 mg/kg, IP)
175 nM at 8 hour
4 nM at 24 hour

Individual and mean diluted blood concentration-time data of (6S+6S)-PEG7 after an IP dose at 50 mg/kg in male CD1 Mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL)			Mean (ng/mL)	SD	CV(%)
			Mouse#1	Mouse#2	Mouse#3			
50	IP	0	BQL	BQL	BQL	BQL	NA	NA
		0.083	697	2043	1141	1294	686	53.0
		0.25	3698	6032	3778	4503	1325	29.4
		0.5	5543	6956	5715	6071	771	12.7
		1	5295	6856	6009	6053	781	12.9
		2	3745	4390	3682	3939	392	9.95
		4	2097	905	358	1120	889	79.4
		8	29.9	461	104	198	231	116
		24	4.65	6.90	3.49	5.01	1.73	34.6
PK parameters		Unit	Mouse#1	Mouse#2	Mouse#3	Mean	SD	CV(%)
T_{max}		hr	0.500	0.500	1.00	0.667	0.289	43.3
C_{max}		ng/ml	5543	6956	6009	6169	720	11.7
Ternimal $t_{1/2}$		hr	2.26	2.78	3.07	2.70	0.411	15.2
AUC_{last}		hr*ng/ml	19153	23231	15243	19209	3994	20.8
AUC_{INF}		hr*ng/ml	19168	23259	15259	19228	4000	20.8



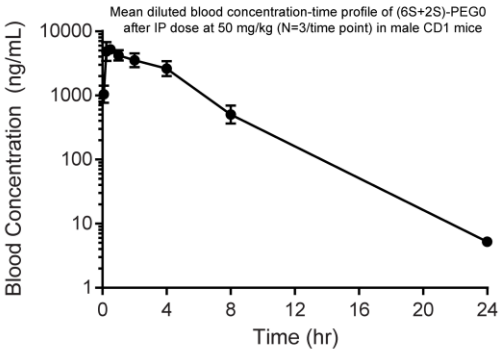
b



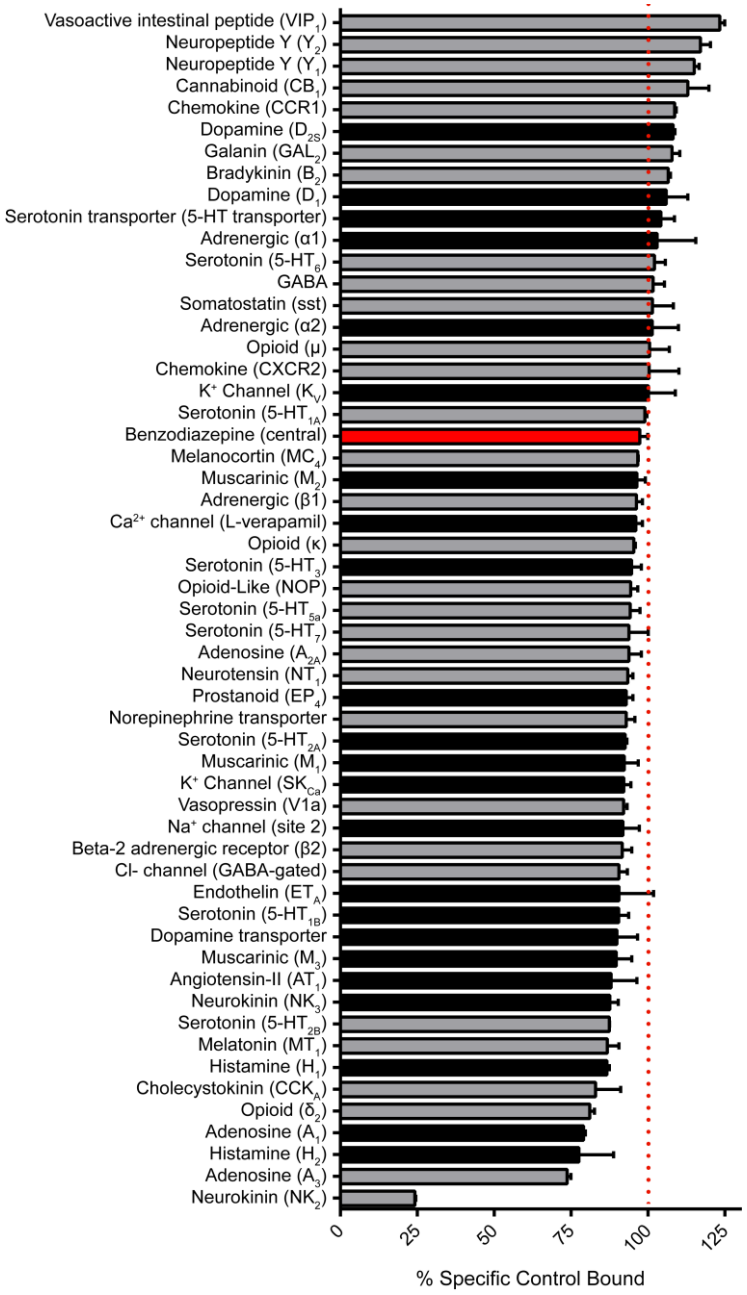
(6S+2S)-PEG0 (6) (50 mg/kg, IP)
576 nM at 8 hour
6 nM at 24 hour

Individual and mean diluted blood concentration-time data of (6S+2S)-PEG0 after an IP dose at 50 mg/kg in male CD1 Mice

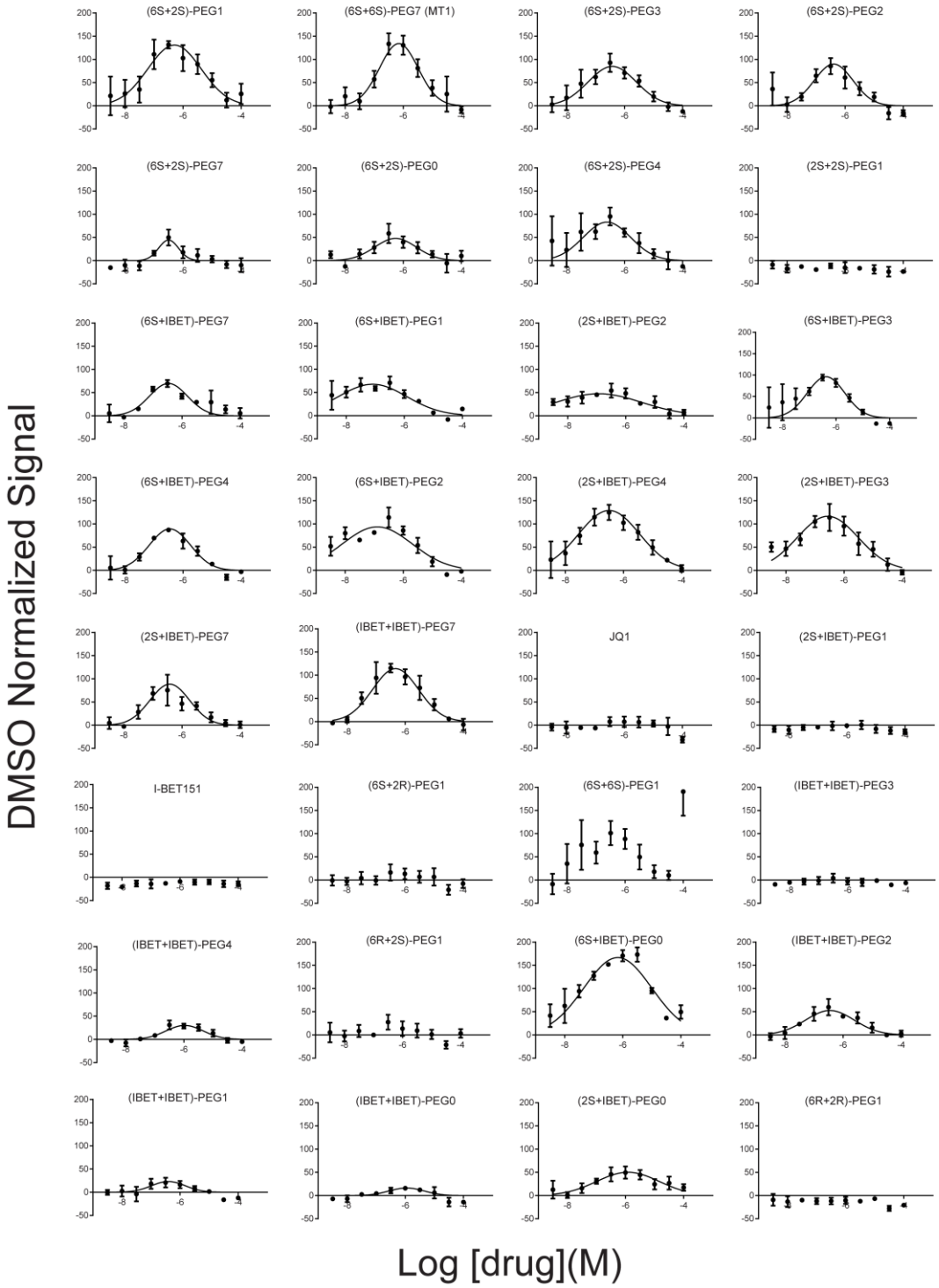
Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL)			Mean (ng/mL)	SD	CV(%)
			Mouse#1	Mouse#2	Mouse#3			
50	IP	0	BQL	BQL	BQL	BQL	NA	NA
		0.083	615	1282	1243	1047	374	35.8
		0.25	4072	3371	7055	4833	1956	40.5
		0.5	5570	4692	5455	5239	477	9.11
		1	4790	3216	4643	4216	869	20.6
		2	3183	2799	4653	3545	979	27.6
		4	3343	1733	2759	2612	815	31.2
		8	593	281	630	501	192	38.3
		24	6.28	5.10	4.26	5.21	1.02	19.5
PK parameters		Unit	Mouse#1	Mouse#2	Mouse#3	Mean	SD	CV(%)
T_{max}		hr	0.500	0.500	0.250	0.417	0.144	34.6
C_{max}		ng/ml	5570	4692	7055	5772	1194	20.7
Ternimal $t_{1/2}$		hr	2.37	2.43	2.17	2.32	0.137	5.92
AUC_{last}		hr*ng/ml	27391	17279	28747	24472	6266	25.6
AUC_{INF}		hr*ng/ml	27412	17297	28760	24490	6266	25.6



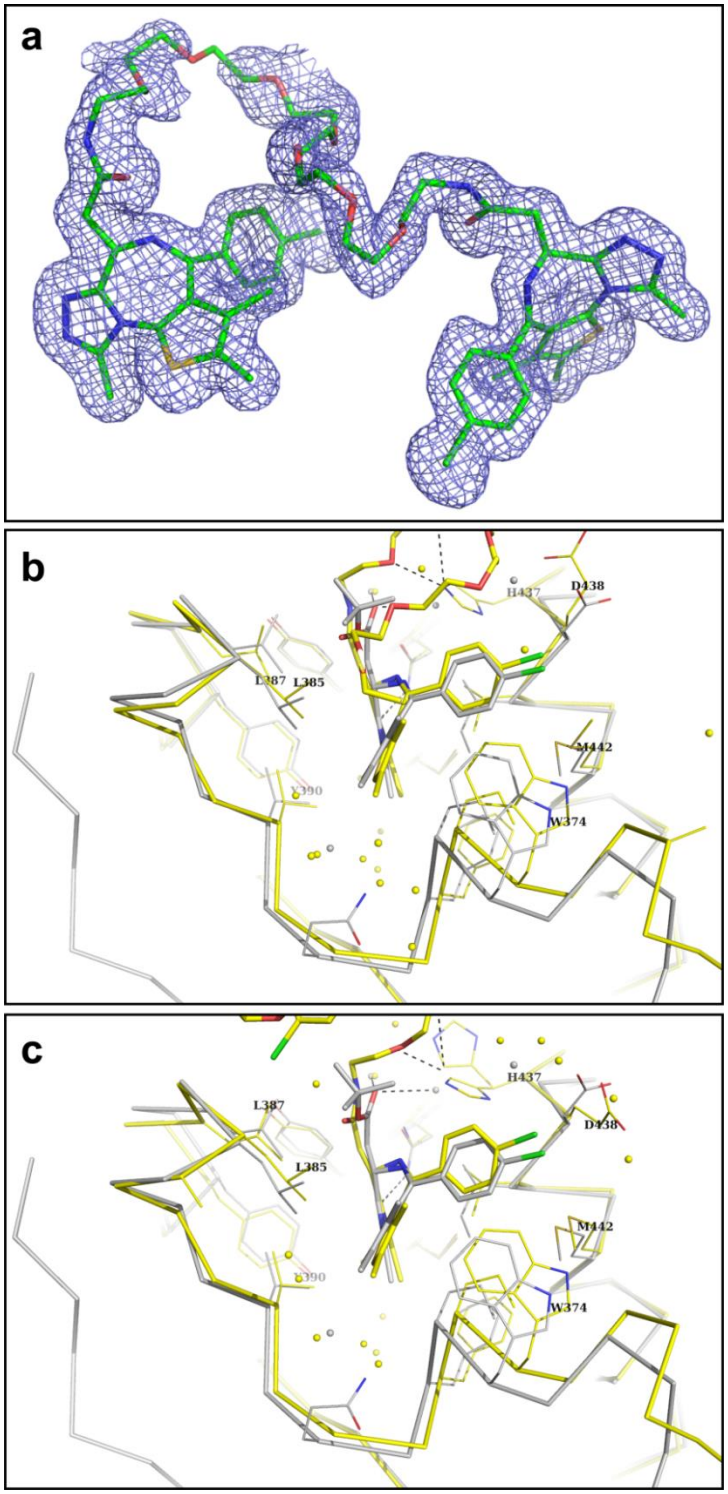
Supplementary Figure 7 | Pharmacokinetic analysis of bivalent inhibitors. (**a, b**) Plasma pharmacokinetic analyses of (a) (6S+2S)-PEG0 (50 mg/kg) and (b) (6S+6S)-PEG7 (50 mg/kg) administered intraperitoneally to CD1 mice (n = 3).



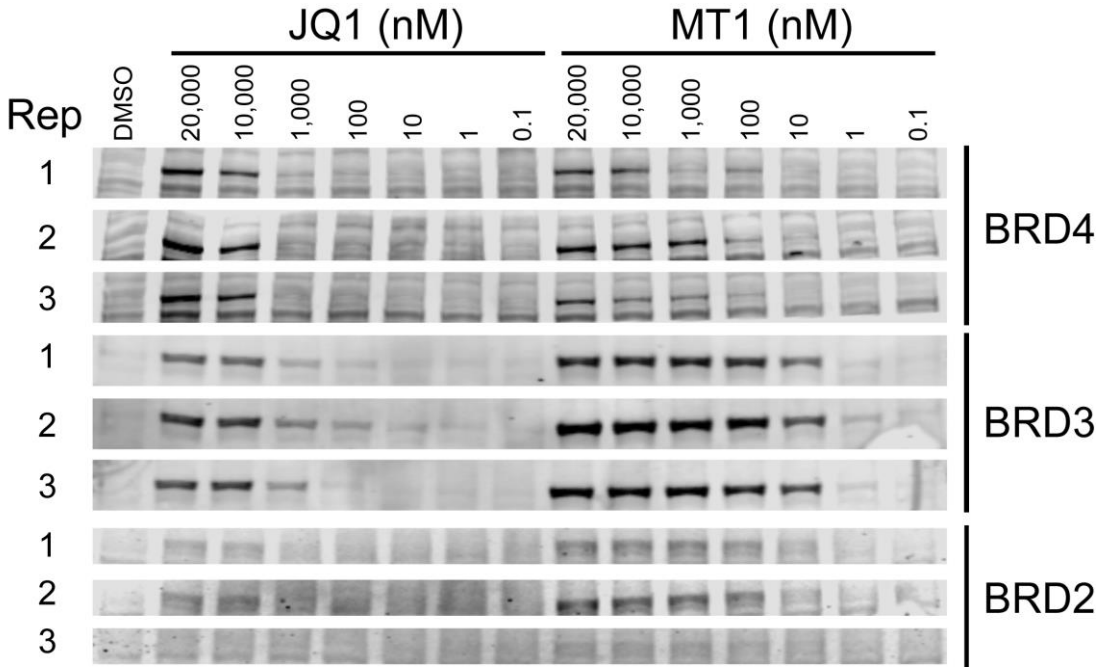
Supplementary Figure 8 | MT1 exhibits limited activity against a panel of human recombinant ligand and ion receptors. MT1 (1 μM) was screened against a panel of 55 ligand receptors, ion channels and transport proteins using an established and widely utilized commercial assay (ExpresSProfile; CEREP). Competitive binding of MT1 against agonists (gray bars) and antagonists (black bars) of the indicated receptors, ion channels and transport proteins are depicted. Competitive binding of diazepam to the central benzodiazepine receptor is highlighted in red. Error bars represent the range of two independent measurements from the mean.



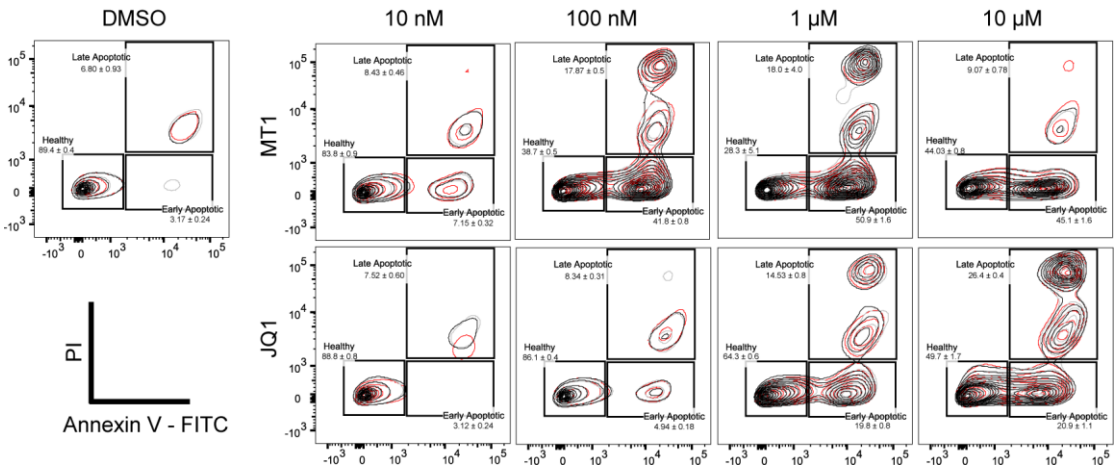
Supplementary Figure 9 | BRD4(1) Dimerization Alpha. Differentially tagged (His and GST) BRD4(1) constructs were incubated in the presence of drug and then glutathione donor and nickel acceptor AlphaScreen™ beads were added to the solution and luminescence was monitored to determine compound mediated dimerization. Data points are in triplicate and represent means ± SD.



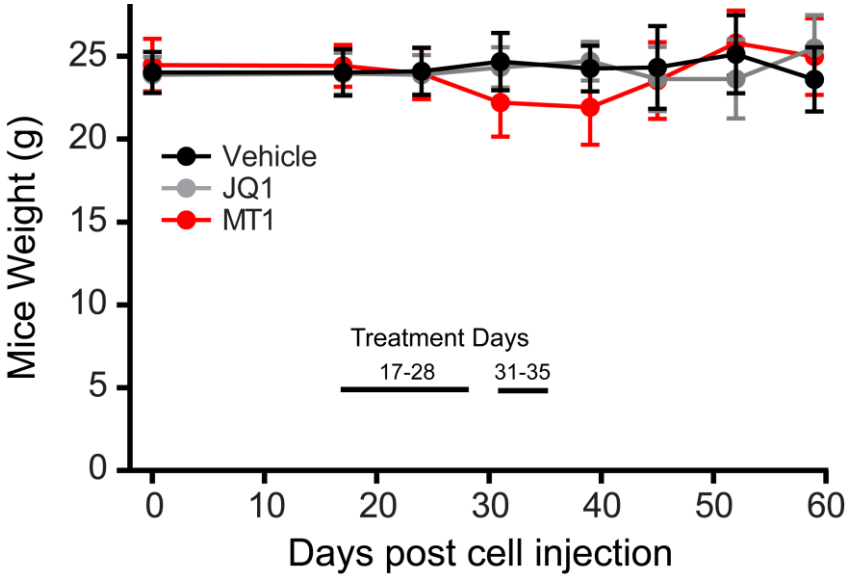
Supplementary Figure 10 | Cocrystal structures of MT1 with BRD4(2). (**a**) An unbiased 2Fo - Fc electron density map around MT1 after the ligand was modeled with sigma level of 0.8. (**b**, **c**) Superpositions between the single binding mode of JQ1/BRD4(1) (PDB: 3MXF, gray) and the two binding modes of MT1 in each binding pocket of the two BRD4(2) (yellow) units crystalized.



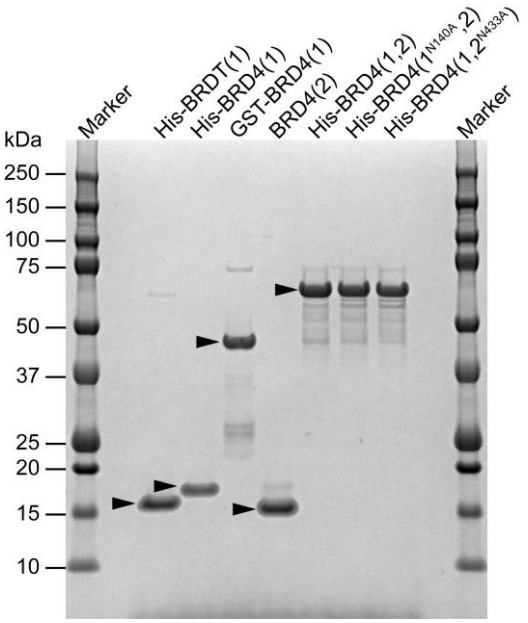
Supplementary Figure 11 | CETSA of JQ1 and MT1. MV4;11 cells were treated with JQ1, MT1, or DMSO and then subject to a heat shock to denature and aggregate proteins. Cells were lysed, clarified by centrifugation and stabilized protein in the supernatant was analyzed by SDS-PAGE and immunoblotting in technical triplicates. Full blots can be found in Supplementary Fig. 15.



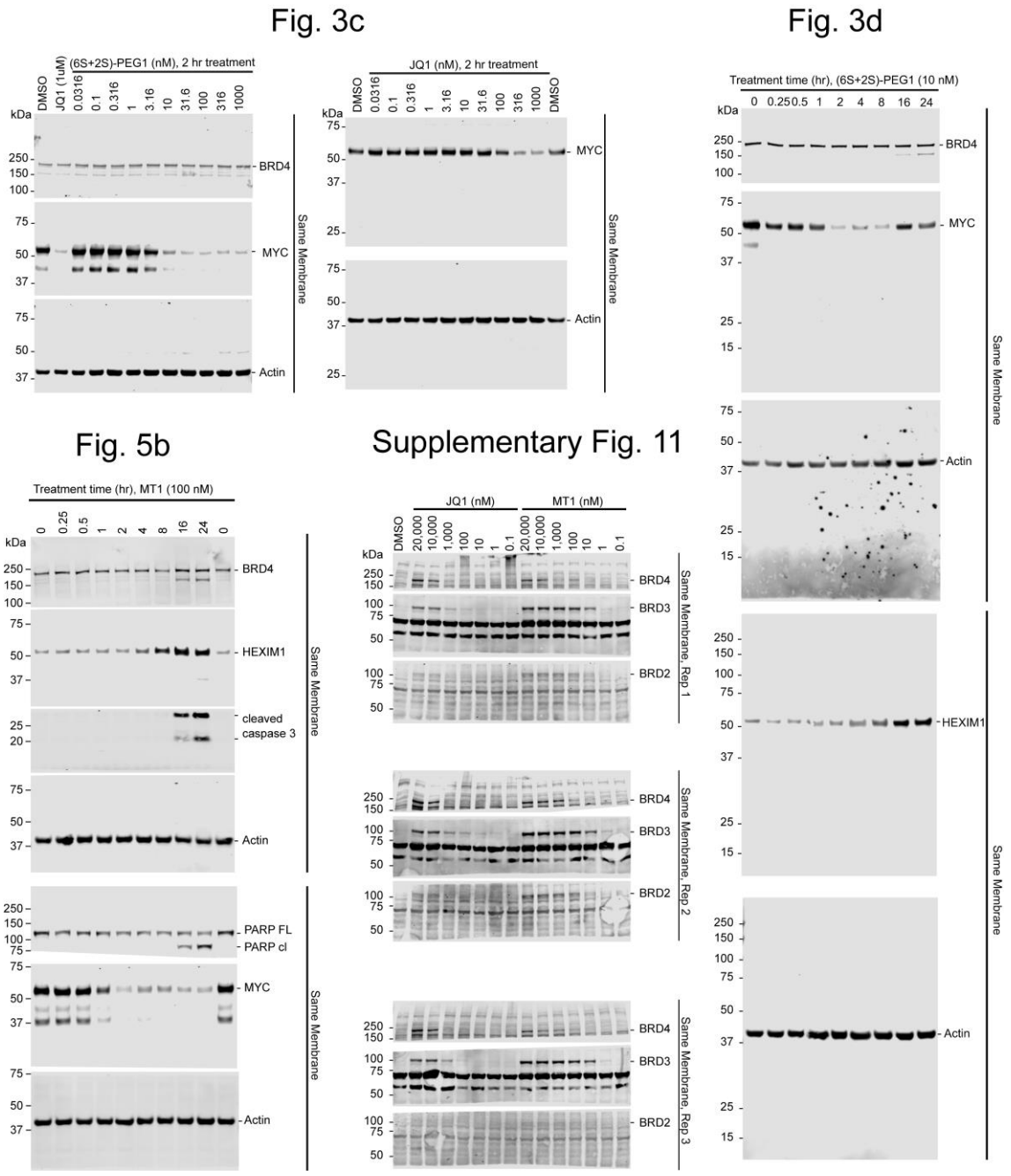
Supplementary Figure 12 | Apoptotic advantage of MT1 over JQ1 by flow cytometry. MV4;11 cells treated with either MT1, JQ1 or DMSO for 24 hours were analyzed for Annexin-V and propidium iodide staining by flow cytometry. Data are displayed as raw contour plots. Gating used here and in figure 5c is shown. Percentages are shown as the mean of three biological replicates ± SD.



Supplementary Figure 13 | Tolerability/toxicity of MT1. In a xenograft model of disseminated leukemia (MV4;11), mice were treated with vehicle (n=11), JQ1 (n=11), or MT1 (n=11) at 44 µmol/kg for the indicated days. Mice were weighed at the indicated times points. Data points represent means ± SD.



Supplementary Figure 14 | Purified proteins gel. 2 µg of each recombinant protein used in this study were run on an SDS-PAGE gel and then stained with Coomassie blue stain. Arrows indicate the major desired protein band.



Supplementary Figure 15 | Full western blots from figures 3c, 3d, 5b , and supplementary figure 11. Doses and time points are indicated above blots. For each blot a loading control from the same membrane has been included. For the CETSA experiment from supplementary figure 11 there are no loading controls.

Supplementary Table 1 | Potency of dimeric compounds for BET family bromodomains.

DiscoveRx Gene Symbol	Kd (nM)		
	(+)-JQ1	(6S+2S)-PEG1	MT1
BRD2(1)	27	0.014	0.16
BRD2(2)	18	0.0069	0.033
BRD2(1,2)	5.6	0.01	0.024
BRD3(1)	14	0.13	0.059
BRD3(2)	19	0.0027	0.07
BRD3(1,2)	14	0.11	0.012
BRD4(1)	14	0.18	0.099
BRD4(2)	8.2	0.0043	0.053
BRD4(1,2)	7.3	0.012	0.087
BRD4 (full-length, short-iso.)	11	0.0078	0.062
BRDT(1)	47	0.29	0.56
BRDT(2)	35	0.066	0.086
BRDT(1,2)	46	0.42	0.076
All others	≥24,000	≥2,500	≥1,800

Supplementary Table 2 | Data collection and refinement statistics (molecular replacement).

	BRD4(2)/MT1 (5JWM)
Data collection	
Space group	C 2 2 21
Cell dimensions	
a, *b*, *c* (Å)	75.67, 107.14, 74.84
α, β, γ (°)	90.0, 90.0, 90.0
Resolution (Å)	43.56 – 1.71 (1.771 – 1.71)*
R_{merge}	0.0459 (0.662)
$I / \sigma I$	16.8 (2.3)
Completeness (%)	99.2 (99.5)
Redundancy	3.6 (3.7)
Refinement	
Resolution (Å)	43.56 – 1.71 (1.771 – 1.71)
No. reflections	32877 (3274)
Rwork / Rfree	0.1498 /0.1759
No. atoms	
Protein	1761
Ligand/ion	77
Water	204
B-factors	
Protein	30.3
Ligand/ion	36.2
Water	40.2
R.m.s. deviations	
Bond lengths (Å)	0.005
Bond angles (°)	0.78

Data were collected on a single crystal that led to this structure.

*Values in parentheses are for highest-resolution shell.